SIX1 mutations cause branchio-oto-renal syndrome by disruption of EYA1–SIX1–DNA complexes

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Urinary tract malformations constitute the most frequent cause of chronic renal failure in the first two decades of life. Branchio-otic (BO) syndrome is an autosomal dominant developmental disorder characterized by hearing loss. In branchio-oto-renal (BOR) syndrome, malformations of the kidney or urinary tract are associated. Haploinsufficiency for the human gene *EYA1*, a homologue of the *Drosophila* gene *eyes absent* (*eya*), causes BOR and BO syndromes. We recently mapped a locus for BOR/BO syndrome (*BOS3*) to human chromosome 14q23.1. Within the 33-megabase critical genetic interval, we located the *SIX1*, *SIX4*, and *SIX6* genes, which act within a genetic network of *EYA* and *PAX* genes to regulate organogenesis. These genes, therefore, represented excellent candidate genes for *BOS3*. By direct sequencing of exons, we identified three different *SIX1* mutations in four BOR/BO kindreds, thus identifying *SIX1* as a gene causing BOR and BO syndromes. To elucidate how these mutations cause disease, we analyzed the functional role of these *SIX1* mutations with respect to protein–protein and protein–DNA interactions. We demonstrate that all three mutations are crucial for Eya1–Six1 interaction, and the two mutations within the homeodomain region are essential for specific Six1–DNA binding. Identification of *SIX1* mutations as causing BOR/BO offers insights into the molecular basis of otic and renal developmental diseases in humans.

Urinary tract malformations constitute the most frequent cause of chronic renal failure in the first two decades of life (1). Branchio-oto-renal syndrome (BOR) (OMIM 113650; www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM) is an autosomal dominant developmental disorder of kidney and urinary tract malformations with hearing loss (2), featuring a wide intrafamilial variability and reduced penetrance (3). Branchio-otic syndrome (BO) is a related disorder without renal anomalies (OMIM 602588). It was demonstrated that BOR and BO can be caused by allelic variants of *EYA1* mutations (4–7). BOR/BO shows a prevalence of 1:40,000 in the general population and is responsible for 2% of profound deafness in children (8). The major feature of BOR is hearing loss (93% of patients), which can be conductive, sensorineural, or both and varies in age of onset (9). Dominant mutations in the human homologue of the *Drosophila eyes absent* gene (*EYA1*) cause BOR/BO type 1 (OMIM 601653) (5). *Eya1*-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia (10). Another member of the *EYA* gene family, *EYA4*, is responsible for late-onset deafness (11). An additional locus (*BOS2*) for BO was localized to chromosome 1q31 (OMIM 120502) (3). We recently mapped a locus (*BOS3*) for BOR/BO (OMIM 608389) to human chromosome 14q23.1-q24.3 (12). Within the 33-megabase critical genetic interval, we located the *SIX1*, *SIX4*, and *SIX6* genes (GenBank accession nos. NM_005982, NM_017420, and NM_007374, respectively), which are known to play a role in the *EYA–SIX–PAX* hierarchy of regulatory genes for the embryonic development of ear, kidney, and other organs (10, 13–16).

Methods

Patients and Families. We obtained informed consent and extracted genomic DNA as described (17) from peripheral whole blood samples of family members with BOR/BO syndrome. The diagnosis of BOR/BO syndrome was based on the following criteria: (*i*) otic defect compatible with BOR/BO syndrome (either sensorineural deafness, conductive, or mixed deafness); (*ii*) segregation compatible with autosomal dominant inheritance; and (*iii*) developmental defect of the kidney or urinary tract compatible with BOR as additional criteria, if it were present.

Mutational Analysis. From a human genomic sequence contig (GenBank accession no. NT_026437), we generated exon-flanking primers to all exons of the human *SIX1*, *SIX2, SIX4*, and *SIX6* genes (Table 2, which is published as supporting information on the PNAS web site). Direct sequencing was performed by using the dideoxy chain termination method on an automated ABI capillary sequencer as described (18). Sequences were evaluated with SEQUENCHER and MUTATIONEXPLORER software.

Plasmids and Site-Directed Mutagenesis of *Six1* Constructs. *Six1* full-length cDNA was cloned into either pGEX-2T vector for producing the GST–Six1 fusion protein, pGBT9 vector for yeast two-hybrid assays, or pcDNA3 vector for cell culture analysis. To introduce the same amino acid substitutions found in BOR/BO patients into the mouse Six1 protein (GenBank accession no. XP_138167), PCR-based mutagenesis was performed by using the QuikChange mutagenesis kit (Stratagene). Once mutant colonies were identified, the plasmid DNA was isolated and sequenced through the mutation-containing region. Three mutant cDNAs (R110W, Y129C, and delE133) were constructed in the same manner in either pGEX-2T, pGBT9, or pcDNA3 vector.

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Abbreviations: BOR, branchio-oto-renal syndrome; BO, branchio-otic syndrome; Eya1D, Eya1 domain; β-gal, β-galactosidase; CMV, cytomegalovirus; HD, homeodomain; SD, six domain.

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Table 1. Clinical characteristics and mutations detected in *SIX1* in families with BOR/BO syndrome

Family (individual)	Branchial defect	Otic defect	Renal defect	Nucleotide change*	Amino acid residue change	Exon
F1038†	+	+	−	A386G	Y129C	1
F1120	+	+	+	delGGA397–399	delE133	1
K6/7	+	+	−	C328T	R110W	1
S2120	+	+	ND	C328T	R110W	1

ND, no data available

*All mutations were present in the heterozygous state and were absent from at least 90 healthy control subjects.

†This is the kindred that was used for mapping of the locus (12).

Yeast Two-Hybrid Assays. The Matchmaker GAL4 two-hybrid system (Clontech) was used for yeast interaction assays. Six1 was fused with the GAL4 DNA-binding domain of pGBT9 (Gal4BD–Six1). The Eya1 domain (Eya1D) region was fused with the GAL4 activation domain of pGAD424 (Gal4AD–Eya1D). Small-scale LiAc cotransformations of the plasmids into SFY526 cells, β-galactosidase (β-gal) colony-lift filter, and liquid culture assays were performed as described in the Clontech protocols.

Gel-Mobility Shift Assay. A 181-bp fragment of the DNA fragment (*Bam*HI–*Eco*RI fragment of the myogenin luciferase reporter pGL3–6×MEF3) containing six repeats of the myogenin MEF3 site TATGTCAGGGGCTTCAGGTTTCCCTA (19) was labeled with [^{32}P]dATP and used as a probe. GST-fusion proteins of Six1 wild type or its mutants were induced by adding 1 mM isopropyl β-D-thiogalactoside (IPTG), then purified on glutathione–agarose beads (Molecular Probes) and eluted from the beads following the manufacturer's protocol. Ten micrograms of the purified GST-fusion proteins and 0.1 ng (1×10^4 cpm) of the labeled probe were mixed as described (20). GST alone was used as a negative control.

Transfection and Luciferase Assay. The human embryonic kidney HEK293 cell line was cultured as described (21). The myogenin luciferase reporter pGL3–6×MEF3 was used as a reporter (19). One microgram of pGL3–6×MEF3 plasmid was cotransfected with 1 μg of pcDNA3-Six1 wild type or its mutant plasmids, 1 μg of Eya1 alone, or both Six1 and Eya1 together by using FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. Cells were grown in 6-cm-diameter dishes and were additionally cotransfected with 0.25 μg of the cytomegalovirus (CMV) promoter–β-gal (pCMVβ-gal) as an internal control. Forty-eight hours after transfection, cell extracts were prepared and assayed for luciferase activities.

Results

Patients with BOR/BO Exhibit Mutations in *SIX1*. Because we located the *SIX1*, *SIX4*, and *SIX6* genes in the critical genetic region for *BOS3* (12), they represented excellent candidate genes for *BOS3*. Therefore, we performed examinations by directly sequencing all exons of the *SIX1*, *SIX4*, and *SIX6* genes in individuals with BOR/BO from 90 different families and in all 18 individuals with BOR/BO from an Australian kindred (F1038, Table 1), in which we identified the *BOS3* locus (12). We also examined all exons of the *SIX2* gene, because *SIX2* is closely related to the *SIX1* gene family (22). Clinical phenotypes in the individuals of family F1038 have been published and comprised hearing loss (sensorineural, conductive, and mixed) in all 18 individuals, ear pits in six, branchial cysts in three, and lacrimal duct stenosis in three individuals (12). Two patients developed a renal carcinoma. In family F1120, of Swiss descent, the patient described here (Table 1) has a solitary left hypodysplastic kidney with vesico-ureteral reflux and progressive renal failure. This case was published previously as patient IV:5 in a pedigree of "non-syndromic hearing loss" (DFNA23) (23). In family K6/7, which is from German descent, the two affected individuals had bilateral ear pits, branchial cysts, and hearing loss. In family S2120, which is from German/Irish descent, the clinical phenotype of mother and child included hearing loss, preauricular pits, and cup ear deformity. The child also had bilateral branchial cysts.

By direct sequencing of all exons of the *SIX1*, *SIX2*, *SIX4*, and *SIX6* genes in a total of 91 families with BOR/BO syndrome, we identified three different likely loss-of-function mutations in *SIX1* in four different BOR/BO families (F1038, F1120, K6/7, and S2120), in whom *EYA1* mutations had been excluded (Table 1 and Fig. 1). In the kindred that was used for the chromosomal localization of the *BOS3* locus (F1038) (12), we detected a missense mutation of A386G that resulted in an amino acid exchange of tyrosine 129 to cysteine (Y129C) (Table 1 and Fig. 1*A*). This tyrosine is conserved among the *Six1* gene products of mouse and human and the *Drosophila sine oculis* (*so*) gene product (Fig. 2). The amino acid exchange is localized just

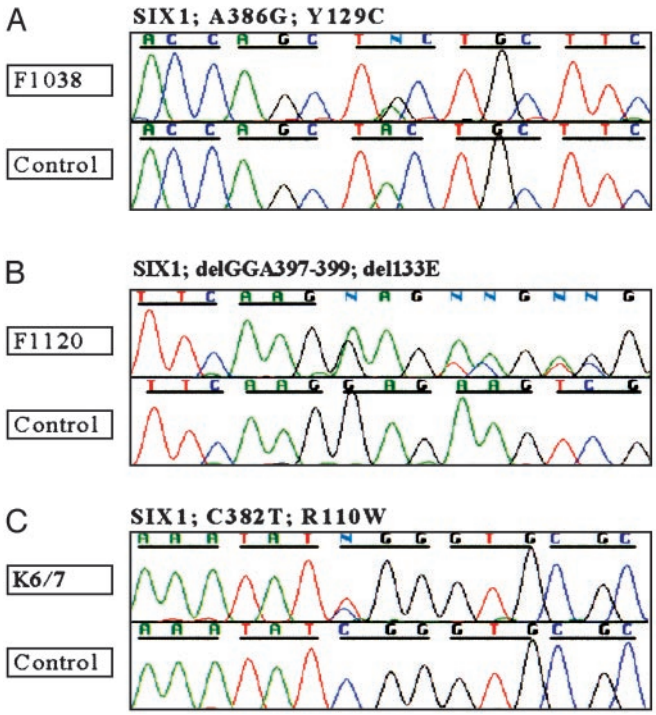

Fig. 1. Loss-of-function mutations detected in exons of the *SIX1* gene of BOR/BO families F1038 (*A*), F1120 (*B*), and K6/7 (*C*), shown in relation to normal controls.

Fig. 2. *SIX1* mutations identified in BOR/BO patients affect protein–protein and protein–DNA interactions. Alignment of the Six-domain (SD) and homeodomain (HD) regions of mouse Six1, human SIX1–6, and the fly So proteins (GenBank accession nos. XP_138167, NP_005973, and NP_476733, respectively). Note that only a partial sequence of the SIX5 cDNA has been deposited in the databases. The tetrapeptides subdividing Six family proteins are boxed (22). Three *SIX1* mutations (R110W, Y129C, and delE133) identified from BOR patients are circled. Amino acid position is numbered according to human SIX1 protein sequence. R110 and E133 are common to all Six proteins isolated so far, and Y129 is also found to be highly conserved among the *Six1* gene products as well as the *Drosophila so* gene product.

N-terminally to the six-type homeodomain (HD) and is the fourth amino acid of the tetrapeptide that was identified as essential for subclassification of Six-family proteins (22). There was full cosegregation of this mutation, with the BOR/BO phenotype within this pedigree. In addition, in family F1120, we detected an in-frame deletion of three nucleotides (delGGA397–399), resulting in a deletion of glutamate 133 (delE133) (Table 1 and Fig. 1*B*). This glutamate is also localized within the N-terminal part of the six-type homeodomain and is identical in all *Six* gene family members isolated so far (Fig. 2). Both amino acid residues Y129 and E133 are located in helix 1 between R5 and Q11 that are typical of most homeodomains but are absent from six-type homeodomains (Fig. 5, which is published as supporting information on the PNAS web site). Furthermore, in families K6/7 and S2120, a point mutation of C328T that resulted in the amino acid substitution of R110W was found (Table 1 and Fig. 1*C*). This arginine residue is located within the Six domain (SD) and is also identical in all *Six* gene family members (Fig. 2). All three *SIX1* mutations were present in the heterozygous state in these BOR/BO patients, but were absent from at least 90 healthy control subjects. No mutations were identified in the *SIX2*, *SIX4*, and *SIX6* genes. In the *SIX4* gene, we only identified a likely polymorphism (G2072A) that led to an exchange of arginine 691 to histidine (R691H). R691 is not conserved in mouse *Six4* sequence. This polymorphism was detected in families K15 and K18, where it did not segregate with the phenotype. This nucleotide exchange was also present in 1 of 83 healthy control subjects. Therefore, it most likely represents a rare polymorphism. In summary, we identified *SIX1* as a gene causing BOR/BO syndrome. Our data suggest that the three amino acid residues of SIX1 R110, Y129, and E133 are essential for the structure or function of the SIX1 protein.

The Three Mutations Detected in BOR/BO Patients Disrupt the Interaction of Six1 with Eya1. In a first attempt to dissect the molecular mechanisms of organ defects detected in the BOR/BO patients, we examined whether these three amino acid alterations identified in the affected patients are crucial for the SIX1 interaction with EYA1. *EYA1* is a member of the *Eya* gene family and mutations in the human *EYA1* cause BOR/BO syndrome (5). Our previous studies have shown that the murine *Eya1* is epistatic to *Six1* in early branchial arch, otic, and kidney development (10, 13, 14) and its gene product physically interacts with Six1 (21). We first introduced the three *SIX1* mutations found in BOR/BO patients into the murine Six1 protein and tested their effect on protein–protein interaction by using the GAL4 yeast two-hybrid system. We fused the Six1 protein and its mutants with a GAL4 DNA-binding domain (Gal4BD–Six1) and used this as the "bait." "Prey" was constructed as fusion with the GAL4 transcriptional activation domain of an Eya1 prey construct that only contained the Eya domain (Gal4AD–Eya1D). Thus, *in vivo* interactions between the bait and prey will result in lacZ transcription. Cotransformation of the Six1 bait construct and the Eya1 prey construct led to strong lacZ expression (Fig. 3*A*). In contrast, the amino acid substitution R110W in SIX1, located in the SD region, resulted in an ≈4-fold decrease in lacZ activity when cotransformed with the Eya1 construct, indicating that this amino acid residue is essential for the Eya1–Six1 interaction (Fig. 3*A*). Similarly, the two SIX1 mutations in the HD domain, amino acid substitution of Y129C and deletion of E133, led to ≈6- and 8-fold decreases in lacZ activity, respectively, when co-transformed with the Eya1 construct, indicating that these two amino acid residues are also crucial for the association of Six1 with Eya1 (Fig. 3*A*).

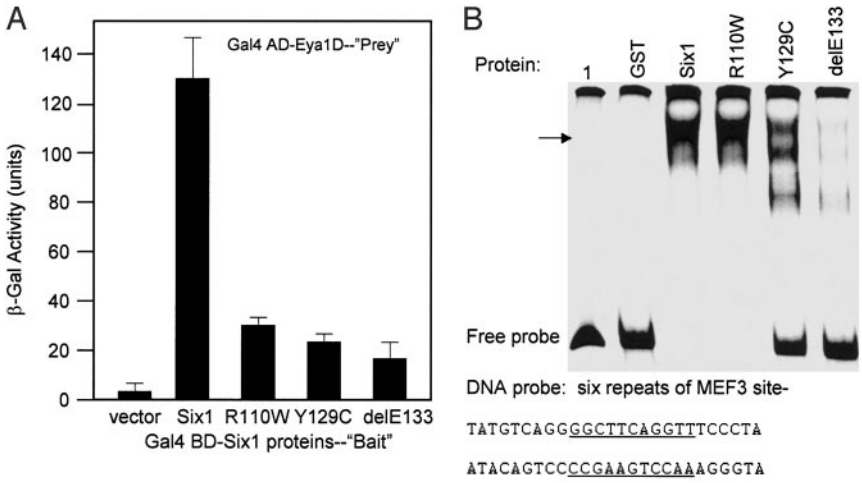

Fig. 3. (*A*) Yeast two-hybrid analysis. Cotransformants were analyzed for their ability to activate lacZ expression by liquid β-gal assay. Cotransformants of Gal4AD–Eya1D prey construct with pGBT9 vector alone was used as a negative control. Strength of interactions was judged by the units of β-gal activity. A result typical of three independent experiments (each performed in triplicate), which yielded essentially the same results, is shown with the standard deviation. (*B*) Gel-mobility shift assay. GST-fusion proteins of Six1 wild type and its mutants were incubated with a labeled multimerized (six copies) MEF3 motif (underlined). GST alone was incubated with the same DNA probe as a negative control. DNA probe alone was loaded onto the gel (lane 1). Arrow points to the shifted complex.

The Two Homeodomain Mutations Interfere with Six1–DNA Binding. We then tested whether these amino acid alterations would affect protein–DNA binding. The Six1 protein has been shown to bind to MEF3 motifs (consensus sequence TCAGGTT) in the myogenin promoter (19). We performed gel-mobility shift assays with a DNA fragment that contains six repeats of MEF3 motifs and with purified GST-fusion proteins of Six1 wild type and the mutants identified in BOR/BO patients. The GST–Six1 wild-type fusion protein efficiently bound to the MEF3 sites (Fig. 3*B*, arrow). The SD mutation R110W did not affect the complex formation of Six1–MEF3. In contrast, the HD mutation Y129C significantly reduced the binding activity of Six1 with MEF3. Similarly, the HD mutation delE133 almost completely abolished the complex formation of Six1–MEF3. Thus, interestingly, the deletion of E133 which results in BOR syndrome showed a stronger effect on the complex formation of both Eya1–Six1 protein–protein interaction (Fig. 3*A*) as well as Six1–MEF3 protein–DNA binding (Fig. 3*B*), compared to the other two mutations that cause BO syndrome. Together, these results indicate that the two HD mutations are crucial not only for Eya1–Six1 protein–protein interaction, but also for Six1–DNA protein–DNA binding.

The Three *SIX1* Mutations also Abolish Reporter Gene Expression in HEK293 Cells. Because previous studies have shown that Eya1 possesses a transcriptional activation function and Eya and Six are able to synergistically activate reporter gene transcription (24, 25), we further assayed the effect of the *SIX1* mutations on the transcription of the myogenin reporter pGL3–6×MEF3 containing six repeats of MEF3 motifs that bind to Six1 in a cell culture system (see *Methods* and ref. 20). Coexpression of Six1 and Eya1 resulted in an ≈3-fold increase in luciferase activity when compared to either Six1 or Eya1 alone (Fig. 4). However, no increase in luciferase activity in HEK293 cells was observed by coexpression of Eya1 with each of the three Six1 mutants. The failure to activate reporter gene expression could be caused by affecting either Six1–Eya1 protein–protein interaction, Six1–DNA binding, or both.

Discussion

In this study, we identified three mutations in the *SIX1* gene as a cause of BOR/BO syndrome. Of the three mutations that we identified as causing BOR/BO, the SD mutation R110W was relatively mild, affecting Eya1–Six1 interaction only, whereas the two HD mutations, Y129C and delE133, showed an effect not only on Six1–Eya1 interaction but also on Six1–DNA binding. Because all *SIX1* mutations detected represent missense mutations and not truncating mutations, the respective transcripts are unlikely to be subject to nonsense-mediated decay. Therefore, they most likely do not represent null alleles. This is supported by the finding that all three Six1 mutant proteins were stable when produced in *Escherichia coli* or *in vitro*. Furthermore, because Eya1–Six1 interaction was also tested directly *in vitro* by yeast two-hybrid

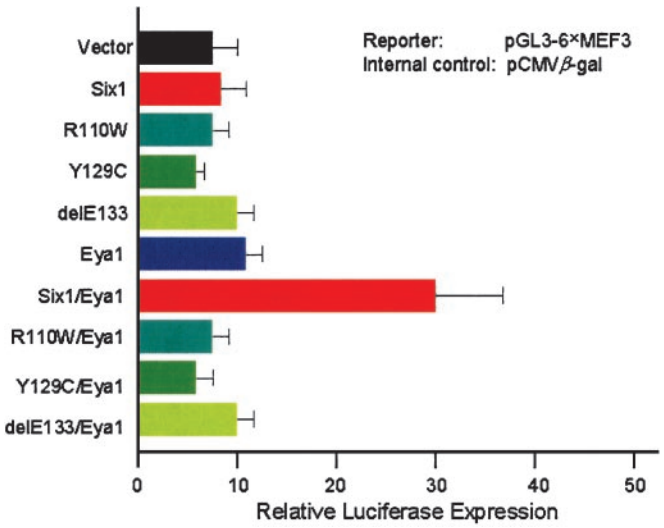

Fig. 4. *SIX1* mutations abolish the activation of myogenin promoter MEF3 by coexpression of Six1 and Eya1. The myogenin luciferase reporter pGL3–6×MEF3 was cotransfected with pcDNA3-Six1, pcDNA3-Six1-R110W, pcDNA3-Six1-Y129C, pcDNA3-Six1-delE133, pFlag-Eya1, or both the Six1 and Eya1 plasmids together in HEK293 cells. Luciferase activity in the cell lysate was normalized with β-gal activity of pCMVβ-gal as an internal control. The activity at each data point is relative to that obtained by the control pCMV vector. The mean fold activation from three independent experiments (each performed in duplicate) is shown with the standard deviation.

GENETICS

assay, which showed strongly decreased interaction (Fig. 3*A*), haploinsufficiency is the most likely molecular mechanism for these mutations. Thus, these results provide important insights into the molecular basis of developmental defects of kidney, ear, and other branchial arch-derived organs, and into the functional mechanisms of BOR/BO syndrome.

The discovery that the *SIX1* gene is responsible for BOR/BO syndrome is strongly supported by recent observations that mice heterozygous for a null (knockout) allele of *Six1* exhibit phenotypes similar to that occurred in BOR/BO syndrome (13, 15, 16). Interestingly, consistent with the feature that hearing loss was observed in all affected individuals, all $Six1^{+/-}$ mice carrying the null in different genetic backgrounds showed hearing loss (16). The renal abnormalities in BOR syndrome were severe in only ≈6% of heterozygotes and include collecting system duplications, renal hypoplasia, dysplasia, and agenesis (8, 26–28). The renal $Six1^{-/-}$ homozygous null phenotype in mice consisted of bilateral renal agenesis in 39 of 40 (97.5%). Only 1 of 40 animals (2.5%) showed severely hypoplastic and dysplastic kidney rudiments (13). In these mice, the inner ear failed to form completely, and outer and middle ears were also malformed (16). If this genotype/phenotype relationship would be conserved in humans, one would expect *SIX1* homozygous null alleles to be present in patients with renal agenesis and ear malformations, who most likely would present with the Potter sequence. Our observation of renal defects at low penetrance in $Six1^{+/-}$ 129S6/SvEv mice is consistent with the previous description of wide variability of BOR syndrome phenotypes even within families: ≈16% of $Six1^{+/-}$ mice in 129S6/SvEv background displayed renal defects, including renal hypoplasia (2 in 19 newborns, ≈11%) and bilateral agenesis (1 in 19 newborns, ≈5%) (13). No renal defect was observed in 12 $Six1^{+/-}$ C57BL/6J newborns analyzed so far (13). This further suggests genetic background effects on the severity of the associated defects in BOR/BO syndrome. Other BOR features, including defects derived from branchial arches, were also observed in *Six1* mutant mice (13, 15, 16).

Our results show that the three *SIX1* mutations identified in BOR/BO patients reduced direct or indirect Eya1–Six1 interactions. These results are also consistent with our recent findings that *Six1* and *Eya1* show synergistic interaction during inner ear and kidney development (13, 16). Interestingly, more detailed molecular and phenotypic analyses of inner ear development in $Eya1^{-/-}$ or $Six1^{-/-}$ embryos detected similar cellular and molecular defects in both mutants (D.S. and P.-X.X., unpublished data). Furthermore, we found that the two HD mutations Y129C and delE133 also markedly reduced Six1–Mef3 protein–DNA binding. Thus, our results, combined with the observation of a similar cellular and molecular mechanism by which Six1 and Eya1 act in inner ear development (D.S. and P.-X.X., unpublished data), indicate that Six1 functions closely together with Eya1 to regulate diverse pathways controlling development of the auditory, renal, and branchial arch systems. Our results also strongly suggest that the three *SIX1* mutations in BOR/BO patients are likely to influence the formation of Eya1–Six1 or Six1–DNA complexes, thus causing the disease phenotype by reducing the expression of downstream genes. Interestingly, we have found that the expression of *Bmp4* and *Fgf3* requires both *Eya1* and *Six1* function during inner ear development (16). We have recently found that, during kidney morphogenesis, *Eya1* acts as a critical regulator for the initiation of kidney organogenesis and that *Eya1*, *Six1*, and *Pax2* interact synergistically to regulate *Gdnf* expression during branching morphogenesis in kidney development (P.-X.X., unpublished data). No obvious downstream target genes have been found to map to the *BOS2* locus (3).

Our observation of renal defects at low penetrance in $Six1^{+/-}$ 129S6/SvEv mice is consistent with the previous description of wide variability of BOR syndrome phenotypes even within families. Interestingly, in family F1120, of Swiss descent, the patient described here (Table 1) has a solitary left hypodysplastic kidney with vesico-ureteral reflux and progressive renal failure, which was previously not recognized when this patient was reported as patient IV:5 in a pedigree of "non-syndromic hearing loss" (DFNA23) (23). It appears, therefore, that DFNA23 could represent part of the BOR phenotype with partial renal involvement. It is currently unknown whether mechanisms of variable expressivity are responsible for kidney involvement in patients with *SIX1* mutations, or whether this association is accidental. These questions will have to be answered once larger numbers of patients with *SIX1* mutations are known. However, our results from *Six1* heterozygous mice suggest that additional modifier genes may exist to influence Six1 activity or function and thereby modulate expression of the *Six1* mutant phenotype. Delineation of genotype/phenotype relationship in BOR/BO syndrome caused by mutations in *SIX1* and related genes will offer direct access to the disease mechanisms involved. Mutations in *SIX1* may represent the underlying diagnosis in a significant number of cases with congenital abnormalities of the urinary tract as well as deafness.

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